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The role of mTor in the pathogenesis of tau-related pathologies in Alzheimer Disease

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The role of mTor in the pathogenesis of tau-related pathologies in Alzheimer disease

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ABSTRACT

An important neurophathological hallmark of Alzheimer disease (AD) is the progressive formation of neurofibrillary tangles composed of aberrant hyperphosphorylated tau aggregates. Evidence from human postmortem AD brains and *in vitro* and *in vivo* rapamycin-treated drug models implicated an abnormal accumulation of the mammalian target of rapamycin (mTor) in AD brains. Previous evidence also indicated that the sequential molecular events, such as the formation and phosphorylation of tau, can be regulated by p70S6 kinase, the well characterized downstream target of mTor. Since mTor is a serine/threonine (S/T) kinase that plays a key role in the regulation of protein homeostasis and degradation, and in cellular functioning, including cell survival, cell growth and proliferation, we chose in our studies to investigate mTor's involvement in the regulation of tau associated pathologies in AD.

In **Paper I** we investigated whether or not mTor mediates tau protein homeostasis. By immunostaining we had found that the active form of mTor aggregated in tangle-bearing neurons in AD brains. Employing mass spectrometry and Western blotting, we identified that mTor directly phosphorylated tau protein at three phosphoepitopes (S214, S356 and T231). We have further developed a variety of stable cell lines with genetic modification of mTor activity using SH-SY5Y neuroblastoma cells as background, confirmed the tau phosphorylation sites found *in vitro*, and found that mTor mediates the synthesis and accumulation of tau, resulting in compromised microtubule stability. The altered mTor activity has been found to cause fluctuation of the level of a battery of tau kinases such as protein kinase A (PKA), v-Aktmurine thymoma viral oncogene homolog-1 (Akt), glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinase 5 (cdk5), and tau protein phosphatase 2A (PP2A).

In **Paper II** we investigated whether mTor mediates cell survival. We used mass spectrometry to identify specific protein expression changes associated with cell survival in SH-SY5Y cells expressing genetically modification of mTor. By inducing cell death in SH-SY5Y cells using moderate serum deprivation, we found that up-regulated mTor complex 2 (mTorC2) increases viable cells by flow cytometry. By employing a combination proteomic method and enrichment analysis we observed that mTor significantly altered expression of several proteins (Peroxiredoxin-5, Thioredoxin-dependent peroxide reductase, Cofilin 1 [non-muscle], Mortalin, Annexin A5, and 14-3-3 protein zeta/delta) involved in mitochondrial integrity, apoptosis, and pro-survival functions.

In **Paper III** we investigated the influence of mTor in tau distribution and secretion. By immunostaining, we found that tau protein was localized within different organelles (autophagic vacuoles, endoplasmic reticulum, Golgi complexes, and mitochondria) in postmortem human AD brain. By employing SH-SY5Y cells stably carrying different genetic variants of mTor, we further found that mTor was responsible for the changed balance of phosphorylated (p-)/ non phosphorylated (Np-) tau in the cytoplasm and different cellular compartments. Up-regulated mTor activity resulted in a significant increase in the amount of cytosolic tau as well as its localization in exocytotic vesicles that were not associated with exosomes to release into extracellular space.

In **Paper IV** we investigated the effects of mTor in relationship to cell growth and proliferation. We found that the suppression of mTor decreases the rate of cell proliferation assessed by WST-1(4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay. By using microarray, we exhibited that mTor knockdown up-regulated 27 genes and down-regulated 49 genes related to the regulation of cell growth and proliferation. Silenced mTor seems to inhibit cell growth and proliferation, by regulating not only the AKT-mTor-S6K signaling pathway, but also directly or indirectly affecting key regulator such as Bcl2, CDK4 inhibitor, various interleukins, or the TGF beta superfamily.

These findings can provide a better understanding of the complex role of mTor involved in the biomechanics of different aspects of tau changes in AD and could contribute to developments of mTor as a novel therapeutic target in the future.

LIST OF SCIENTIFIC PAPERS

I. ***Zhi Tang,*** Erika Bereczki, Haiyan Zhang, Shan Wang, Chunxia Li, Xinying Ji, Rui M. Branca, Janne Lehtiö, Zhizhong Guan, Peter Filipcik, Shaohua Xu, Bengt Winblad, and Jin-Jing Pei.
Mammalian Target of Rapamycin (mTor) Mediates Tau Protein Dyshomeostasis.
The Journal of Biological Chemistry. 2013 May;288 (22)： 15556–15570

II. ****Zhi Tang,*** *Ahmet Tarik Baykal, Hui Gao, Hernan Concha Quezada, Haiyan Zhang, Erika Bereczki, Muge Serhatli, Betul Baykal, Cigdem Acioglu, Shan Wang, Eniko Ioja, Xinying Ji, Yan Zhang, Zhizhong Guan, Bengt Winblad, and Jin-Jing Pei. * These authors have contributed equally to the manuscript.
mTor is a Signaling Hub in Cell Survival: A Mass-Spectrometry-Based Proteomics Investigation.
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III. ***Zhi Tang,*** Eniko Ioja, Erika Bereczki, Kjell Hultenby, Chunxia Li, Xinying Ji, Zhizhong Guan, Bengt Winblad, and Jin-Jing Pei
mTor mediates tau protein localization and secretion: implication for Alzheimer's disease.
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IV. ***Zhi Tang,*** Erika Bereczki, Eniko Ioja, Zhizhong Guan, Bengt Winblad, and Jin-Jing Pei
Microarray–Based genomics identifies mTor as a critical gene expression regulator in SH-SY5Y neuroblastoma cells growth and proliferation.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer disease
Aβ	β-amyloid
APP	amyloid precursor protein
Atg	autophagy-related genes
cdk-5	cyclin-dependent protein kinase-5
CaMKII	Ca2+/calmodulin dependent protein Kinase II
CK	casein kinase
ERK	extracellular signal-regulated kinases
ER	endoplasmic reticulum
GSK3	Glycogen synthase kinase-3
LC3	Microtubule-associated protein light chain 3
JNK	c-Jun N-terminal kinases
MAP	microtubule associated proteins
MBD	microtubule-binding domains
MAPK	mitogen-activated protein kinases
MARK	microtubule affinity-regulating kinases
mTor	mammalian target of rapamycin
NFT	neurofibrillary tangle
PHF	paired helical filaments
PDPK	proline-directed protein kinase
PP	protein phosphatase
PI3K	phosphoinositide 3-kinase
PKA	cAMP-dependent protein kinase
SP	senile plaques
SF	straight filaments
S6K	p70S6 kinase
TPK	tyrosine protein kinases

1 INTRODUCTION

1.1 Alzheimer disease

1.1.1 Alzheimer disease: General characteristics

Alzheimer disease (AD) is the most common form of dementia (50-70% of dementia cases) among the elderly. It is a progressive neurodegenerative disorder mainly characterized by memory loss and cognitive impairment. The most common early symptom is having difficulty remembering recent events. With the development of the disease severe symptoms may occur such as disorientation, mood swings, confusion, more serious memory loss, behavior changes, difficulty speaking and swallowing, as well as walking (Waldemar et al., 2007). Current treatments only ameliorate symptoms. No treatment is known to be available that can stop or reverse the progression of AD. Worldwide in 2010, 35.6 million people are estimated to be living with dementia and the total estimated cost of dementia is 604 billion USD. These numbers are expected to almost double every 20 years, to 65.7 million in 2030 and to 115.4 million by 2050 (Wimo et al., 2013). Thus, AD tragically influences not only patients and their families but is also a heavy and growing economic burden for society.

1.1.2 Neuropathology

Neuropathological hallmarks of AD were first described by the German psychiatrist and neuropathologist, Alois Alzheimer, in 1907 (Alzheimer, 1907). Now it is generally accepted that the following features comprise these hallmarks: 1) atrophy of the brain which is due to synaptic and neuronal loss in the temporal and parietal lobes, and parts of the frontal cortex and cingulate gyrus (Wenk, 2003); 2) neurofibrillary tangles (NFTs) composed of straight filaments (SFs) and paired helical filaments (PHFs), with aberrantly hyperphosphorylated tau being the major component (Grundke-Iqbal et al., 1986); and 3) a large number of senile plaques (SPs) consisting of β-amyloid (Aβ) cleaved from amyloid precursor protein (APP). Regarding the latter, two forms of amyloid plaques are found in the AD brain: neuritic and diffuse plaques. The neuritic plaques consist of extracellular, insoluble, fibrillar Aβ (Aβ40/42) core (Iwatsubo et al., 1994) with infiltration of accumulated activated microglia and reactive astrocytes surrounding by dystrophic neurites (Masters et al., 1985). The diffuse plaques are less dense composed of non-fibrillary forms of Aβ (Aβ42) (Gowing et al., 1994) and are believed to be immature senile plaques termed as “pre-amyloid deposits”. Interestingly Aβ does not correlate with cognition, while the number of NFTs correlate with the severity of dementia and with cognitive decline (Arriagada et al., 1992).

1.2 Tau protein

1.2.1 Tau gene, structure and functions

Tau protein is a microtubule-associated protein, it is more often found in neurons than non-neuronal cells. The tau protein is composed of four regions: the N-terminus, the proline-rich region, the microtubule-binding domains (MBDs) and the C-terminal (Wang and Liu, 2008). The human tau gene is over 100 kb, containing 16 exons and is located on chromosome 17q21 (Neve et al., 1986). The major tau protein in the human brain is encoded by 11 exons: constitutive exons (1, 4, 5, 7, 9, 11, 12, and 13) and alternatively spliced exons (2, 3, and 10) (Goedert et al., 1989; Neve et al., 1986). The tau gene is transcribed to yield different tau mRNA species by alternative splicing, resulting in the production of the different tau isoforms. In the human central nervous system exons 2, 3, and 10 are alternatively spliced to generate six tau isoforms that are differentially expressed during brain development (Andreadis, 2005; Goedert et al., 1989). Exons 9–13 encode four microtubule-binding domains which are located in the C-terminal half of the tau protein. Thus, the alternative splicing of exon 10 yields tau protein isoforms with either three (3R) or four (4R) microtubule-binding repeats (Lee et al., 1989; Lu and Kosik, 2001). The microtubule binding domains bind to microtubules and promote their assembly (Preuss et al., 1997). Phosphorylation of certain residues within the microtubule-binding repeats can impair the interaction between microtubules and tau, giving rise to tau detaching from microtubules (Drewes et al., 1995). The six isoforms further generated by alternative splicing of exons 2 and 3 yield inserts in the N-terminal half of the tau protein (Figure 1). The N-terminal half of tau is known as the projection domain (also called flanking domain), and includes acidic and proline-rich regions. The projection domain is proposed to regulate the spacing between microtubules, effectively targeting tau to microtubules and assisting tau in stabilizing microtubules (Chen et al., 1992; Hirokawa et al., 1988).

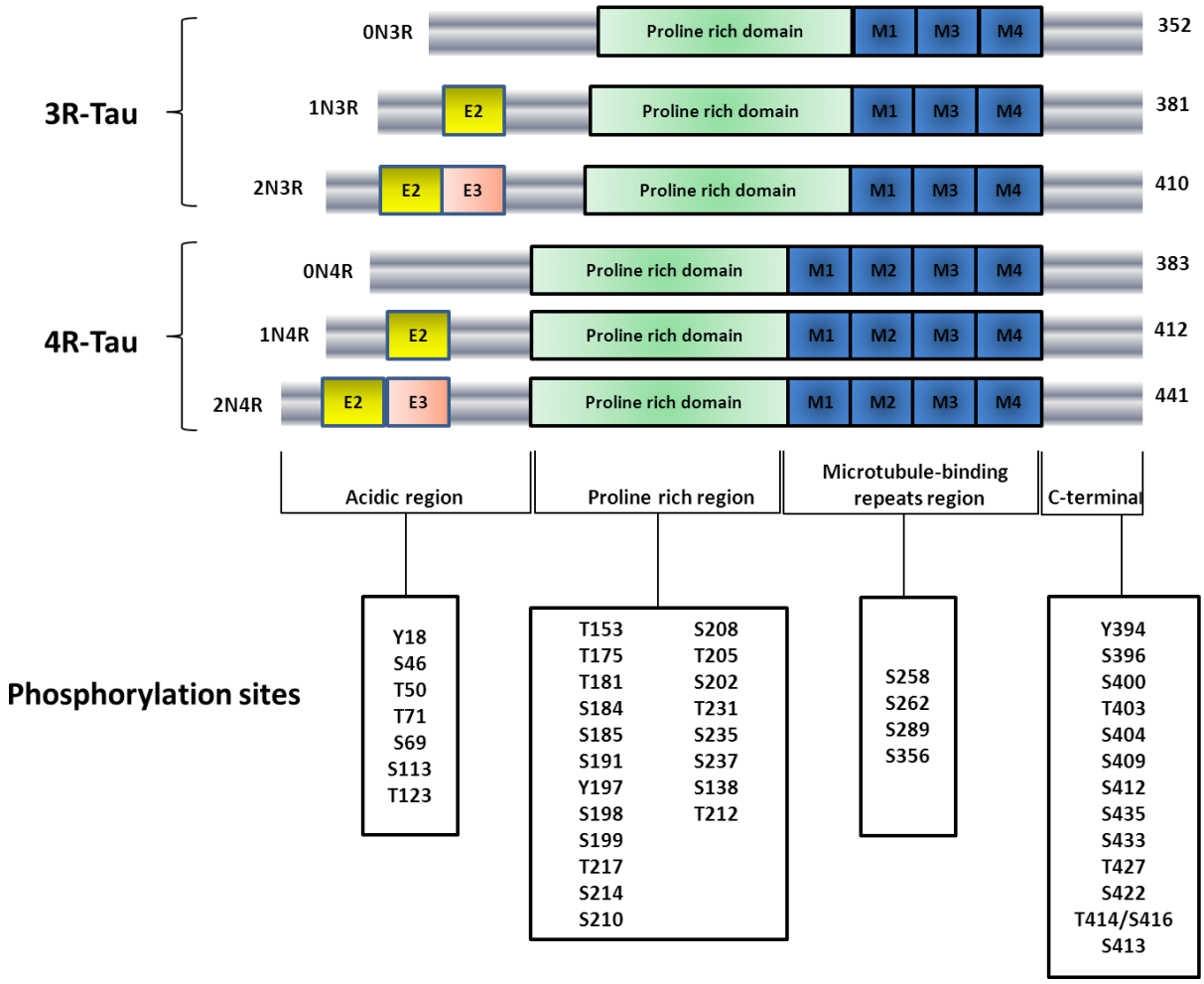

Figure 1. The six tau isoforms and tau phosphorylation sites. Exons 2, 3 and 10 are alternatively spliced giving rise to six isoforms of human CNS tau. Exons 2 and 3 (E2 and E3) encode two different inserts located in the N-terminus of tau. Absence of E2 and E3 gives rise to 0N (N-terminus) tau isoforms, whereas, insertion of E2 produces 1N and insertion of both E2 and E3 results in 2N tau isoforms. M1–M4 represent the four microtubule-binding domains, M2 being encoded by exon 10. Lack of M2 results in the formation of 3R tau, and M2 insertion forms 4R tau isoforms. The proline-rich domain in the center of the tau protein is indicated. Approximately 45 phosphorylation sites from Alzheimer brain have been identified. Adapted from (Noble et al., 2013; Wang and Liu, 2008)

1.2.2 Tau phosphorylation

Several tau post-translational modifications have been described for tau protein including phosphorylation (Grundke-Iqbal et al., 1986; Mandelkow and Mandelkow, 1998), ubiquitination (Mori et al., 1987), glycosylation (Ledesma et al., 1994), oxidation (Troncoso et al., 1993) and truncation (Kovacech and Novak, 2010). The most extensively studied of these has been phosphorylation. Tau is a phospho-protein that contains 85 potential serine (S), threonine (T), and tyrosine (Y) phosphorylation sites on its longest isoform (2N4R). Many phosphorylation sites on tau protein have been found in the proline-rich regions and the

microtubule-binding regions. Employing mass spectrometry and specific antibody staining has enabled 10 phosphorylation sites to be shown on soluble tau obtained from non-dementia brain (Hanger et al., 2007). In contrast, about 45 different serine, threonine and tyrosine phosphorylation sites have been identified in insoluble tau from AD brain (Noble et al., 2013) (Figure 1). Some of these phosphorylation sites such as T181, T231, S396 and S404 have been used as diagnostic biomarkers for AD patients (Blennow, 2004); however, it is still unclear which sites would be the best drug targets.

1.2.3 Tau protein kinases and phosphatases

It has been reported that tau phosphorylation is regulated by a large number of different kinases and phosphatases, and an imbalance of their activity would result in tau hyperphosphorylation in AD (Wang et al., 2007). Tau kinases can be divided into three groups: proline-directed protein kinases (PDPKs), non-PDPKs and tyrosine protein kinases (TPKs). PDPKs are kinases that phosphorylate tau on serine and threonine. These PDPKs include Glycogen synthase kinase-3 (GSK3) (Pei et al., 1999; Pei et al., 1997), cyclin-dependent protein kinase-5 (cdk-5) (Pei et al., 1998) and mitogen-activated protein kinases (MAPK) family, further comprised of the extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinases (JNK) (Atzori et al., 2001; Ferrer et al., 2001; Pei et al., 2002). The non-PDPK group includes casein kinase 1 (CK1) (Hanger et al., 2007), tau-tubulin kinase 1/2 (TTBK1/2) (Houlden et al., 2007), microtubule affinity-regulating kinases (MARKs) (Matenia and Mandelkow, 2009), cAMP-dependent protein kinase (PKA) (Litersky et al., 1996), protein kinase B (PKB/Akt) (Zhou et al., 2009), protein kinase C (PKC) (Alkon et al., 2007) and Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Means, 2000). The third group of kinases, the TPKs can phosphorylate tau protein on 5 tyrosine residues, located at 18, 29, 197, 310 and 394 sites (Wang and Liu, 2008). Proto-oncogene tyrosine-protein kinase (Fyn) (Lee et al., 2004), spleen tyrosine kinase, a receptor tyrosine kinase (Met), and a non-receptor tyrosine kinase (c-Abl) belong to TPKs group (Lebouvier et al., 2008; Lebouvier et al., 2009). Compared with protein kinases, a few phosphatases have been identified to dephosphorylate tau proteins *in vitro* and/or *in vivo*, including protein phosphatase-1 (PP1), PP2A, and PP5 (Liu et al., 2005).

1.2.4 Cellular and subcellular localization of tau

Tau is mainly an intraneuronal protein, it interacts with plasma membrane (Brandt et al., 1995; Pooler et al., 2012), endoplasmic reticulum (ER) (Waterman-Storer et al., 1995) and Golgi network (Farah et al., 2006). The interaction between tau and plasma membrane could be modulated by tau phosphorylation in neural cells (Ekinci and Shea, 2000). Plasma

membrane-associated tau is dephosphorylated at several sites known to be abnormally phosphorylated in AD brain (Arrasate et al., 2000; Ekinci and Shea, 2000; Pooler et al., 2012). Besides tubulin, tau also binds to other proteins, such as spectrin (Carlier et al., 1984), actin (Correas et al., 1990; Griffith and Pollard, 1982), PP1 and PP2A (Liao et al., 1998; Sontag et al., 1999), cdk5 (Sobue et al., 2000), presenilin1 (PS1) (Hendriks et al., 1998), GSK-3β (Pei et al., 1999), α-synuclein (Jensen et al., 1999), phospholipase C-γ (Hwang et al., 1996; Jenkins and Johnson, 1998), and the fyn tyrosine kinase (Klein et al., 2002; Lee et al., 1998). Tau is also found in nuclei, synapses, dendrites and in the extracellular space (Avila, 2010; Noble et al., 2013). In disease states, the localization of tau protein can be altered. The finding of redistribution of hyperphosphorylated tau to the somatodendritic compartments is especially considered as a pathological marker during early development of tau－related pathologies (Andorfer et al., 2003; Braak et al., 2011).

Tau protein plays an important role in neuronal processes including cell division, cell morphology, and intracellular transport (Kolarova et al., 2012; Mandelkow and Mandelkow, 1998). In cells, microtubules serve as tracks for organelle transport regulated by microtubule-dependent motor proteins (Brady and Sperry, 1995). The motor proteins transport their cargoes, such as mitochondria (Morris and Hollenbeck, 1993), lysosomes (Hollenbeck and Swanson, 1990) and exocytotic vesicles (Scales et al., 1997).

1.3 Tau pathologies in Alzheimer disease

Tau is abnormally hyperphosphorylated in AD, which is caused by an imbanlance of tau phosphorylation (Iqbal et al., 2010). The altered tau can disrupt microtubule stability, resulting in daunting changes in the neuronal microtubule network (Figure 2).

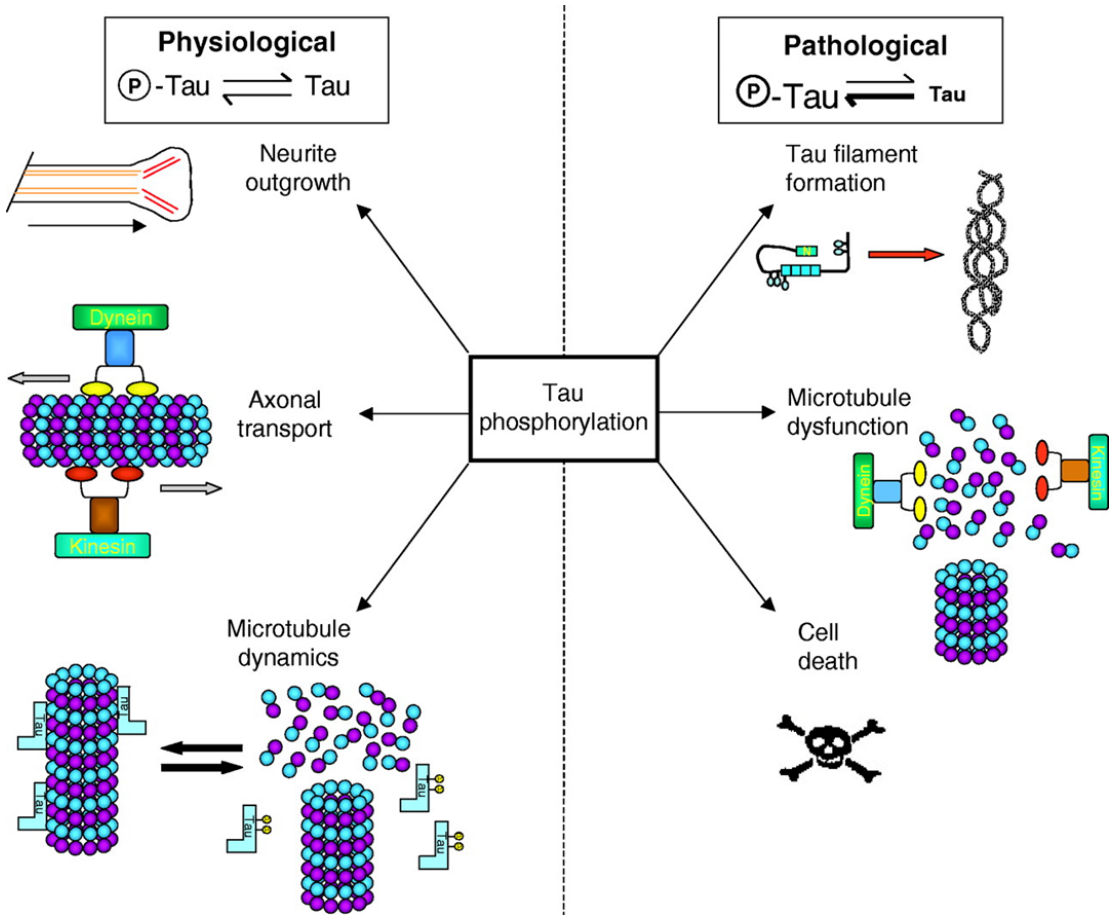

Figure 2. The consequence of an imbalance of tau phosphorylation in Alzheimer disease. Under normal physiological conditions, tau plays a role in regulating neurite outgrowth, axonal transport, and microtubule dynamics and stability. However, in pathological conditions of AD, abnormal tau phosphorylation can cause tau filament formation, decrease microtubule binding to disrupt microtubule stability and, perhaps, even lead to cell death. Originated from (Johnson and Stoothoff, 2004)

1.3.1 The hyperphosphorylation of Tau Protein

At least three- to four-fold more hyperphosphorylated tau has been found in the brains of AD patients compared to non-demented individuals (Iqbal et al., 2010). Increased tau hyperphosphorylation can cause tau protein to lose its biological activity and function. In AD, the abnormally hyperphosphorylated tau could result from up-regulated tau kinases and/or down-regulated tau phosphatases. At least 45 sites, mainly serine or threonine, are found to be abnormally phosphorylated in AD (Noble et al., 2013). The kinases that are thought to play the most important role in tau phosphorylation of the brain include GSK-3β, cdk5, PKA, PKB, S6K, and CaMK-II (Pei et al., 1999; Pei et al., 1998; Pei et al., 2003b; Wang et al., 2007) (Figure 3). GSK-3β may regulate tau phosphorylation in both physiological and pathological situations, and it can phosphorylate tau on Ser199, Thr231, Ser396, Ser400, Ser404, and Ser413 sites both *in vivo* and *in vitro*. These residues are mostly phosphorylated in PHF–tau (Liu et al., 2002). Tau phosphorylation at Ser214 by PKB blocks PKA activity,

whereas tau phosphorylation by GSK-3β efficiently blocks PKB activity at Ser214 (Ksiezak-Reding et al., 2003). GSK-3β or other kinases further phosphorylate tau at Thr231, causing conformational change (Cho and Johnson, 2004). On the other hand, a complementary effect is noticed for PP1 and PP2A, which dephosphorylates tau protein *in vitro*, the activity of them is reduced in AD brains (Liu et al., 2005; Wang et al., 1995). Our group found that S6K could phosphorlate tau at Ser214, Ser262 and Thr212 sites (Pei et al, 2006).

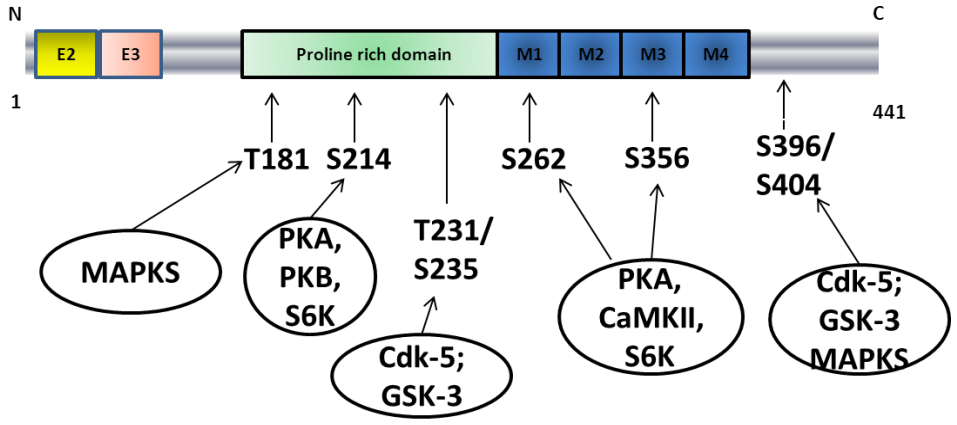

Figure 3. Schematic diagram of the phosphorylation sites on tau protein from human brain. These sites are phosphorylated selectively by key kinases. Adapted from (Buee et al., 2000; Churcher, 2006)

1.3.2 Tau filament formation

Tau is a natively unfolded and soluble protein, however, tau can form insoluble aggregates in AD. Investigation of the role of differently charged regions of tau in self-assembly into SF/PHF found that the β-structure concentrates in the second and third microtubule repeats R2 (275VQIINK280) and R3 (306VQIVYK311) of tau, which can self-assemble into filaments (von Bergen et al., 2000) and co-assemble with heparin (Arrasate et al., 1999). Both the N-terminal and the C-terminal flanking regions to the microtubule binding repeats in normal tau appear to inhibit its self-assembly into filaments. By contrast, in AD, the phosphorylation of the N-terminal and the C-terminal flanking regions results in the formation of SF/ PHF (Alonso et al., 2001). Several early studies have shown that bacterially expressed non-phosphorylated 3R-tau fragments can assemble into PHF-like filaments *in vitro*, although PHFs from AD brain are also proven to be composed of full-length tau (Crowther et al., 1994; Wille et al., 1992). Subsequent studies showed that high concentrations of tau were required for tau polymerization (Crowther et al., 1994; Wille et al., 1992), suggesting that other factors are likely necessary to facilitate tau assembly. Indeed, sulfoglycosaminoglycans (sGAGs), such as heparin, were found to induce phosphorylation of tau by a number of protein kinases and promote fibrillization of full-length tau (Arrasate et al.,

1997; Goedert et al., 1996). Furthermore, RNA (Kampers et al., 1996) and arachidonic acid (Wilson and Binder, 1997) were also shown to facilitate full-length recombinant tau assembly. Moreover, tau filaments assembly may occur in a nucleation dependent manner (Friedhoff et al., 1998).

1.3.3 Tau protein assembly into filaments: the effects of hyperphosphorylation

Abnormal hyperphosphorylation is a major biochemical abnormality of PHF–tau (Iqbal et al., 2005). Tau hyperphosphorylation is likely to be an early event involved in the generation of PHF-tau from normal soluble tau (Wang and Liu, 2008), and it is believed that appearance of the aberrantly hyperphosphorylated tau precedes the formation of NFTs in AD. Iqbal's group found that AD P-tau self-aggregates into PHF-like structures *in vitro* within 90 min, *in vitro* dephosphorylation of AD P-tau could inhibit its self-association into PHF. Furthermore, hyperphosphorylation of each of the six recombinant tau isoforms facilitate their self-assembly into tangles of PHF and SF (Alonso et al., 2001). Avila's group showed that in cultured human neuroblastoma SH-SY5Y cells, it is phosphorylated but not nonphosphorylated form of tau protein that promotes tau filaments formation in the presence of okadaic acid, a phosphatase inhibitor (Perez et al., 2002). Overexpression of human tau in Drosophila could not induce formation of NFTs, while Drosophila that overexpress human tau and GSK-3 or cdk-5 increased tau phosphorylation and NFT formation. Treatment of mutant human tau (P301L) transgenic mice with LiCl, a GSK-3 inhibitor showed a reduction of phosphorylated tau, which is associated with a decreased level of the aggregated tau (Noble et al., 2005; Perez et al., 2003). These studies suggest that the abnormal hyperphosphorylation of tau precedes its accumulation in the affected neurons (Wang and Liu, 2008).

1.3.4 Microtubule disruption

The main function of tau is to bind and stabilize microtubules. Microtubule disruption induced by tau hyperphosphorylation leads to a breakdown in intracellular traffic. The interaction between tau and microtubules depends on the tau microtubule-binding repeats regions, and the projection regions. The C-terminal repeat regions of the longest human tau isoform, with 441 amino acids, 2N4R, are defined as microtubule-binding domains, including repeat 1(R1) (Q244-K274), repeat 2 (R2) (V275-S305), repeat 3 (R3) (V306-Q336) and repeat 4 (R4) (V337-N368). They are the functional domains to promote microtubule assembly and stability. The interaction is regulated by the status of tau hyperphosphorylation (Alonso et al., 2010; Alonso et al., 1997). Specific phosphorylated sites of tau in these

domains affect tau's function. Most notably, in situ phosphorylation of both Ser262 site at R1 and Ser356 site in R4 dramatically lowers the affinity of tau binding to microtubules and mediates the development of cell processes (Biernat and Mandelkow, 1999). It is also clear that phosphorylation in the flanking regions can strongly influence the ability of tau microtubule-binding and microtubule assembly. Phosphorylation at the Ser262 site speeds up the process of tau assembly (Zhou et al., 2006), pseudophorylating tau at the Ser262 site not only leads to lose normal function but also results in microtubule distruption and neuron death (Pei et al., 2006; Zhou et al., 2008). Phosphorylation at the Thr231 site, located in the proline-rich flanking domain, efficiently decreases the ability of tau to bind microtubules in situ; and it also inhibits the ability of tau to stabilize microtubules (Sengupta et al., 1998). Furthermore, when cell lysates were separated into soluble cytosolic and insoluble fractions, almost all the phosphorylated Thr231 tau was present in the soluble fraction (Cho and Johnson, 2003; Hamdane et al., 2003). In contrast, phosphorylated tau at Ser396/Ser404, located in the C-terminal proline-rich domain, did not significantly affect tau binding to microtubules (Cho and Johnson, 2003). Thus phosphorylation of Thr231 seems to play a key role in regulating tau function. Other phosphorylation sites, such as Ser205, Ser212, Ser214 and Ser235, have been shown to affect microtubule association, resulting in neurodegeneration (Cho and Johnson, 2004; Haase et al., 2004; Leger et al., 1997; Sun and Gamblin, 2009; Trinczek et al., 1995). Evidence has shown that tau phosphorylation at Ser214 greatly diminishes tau-microtubule binding *in vitro*, inhibiting microtubule assembly or nucleation, while tau detached from microtubules mediated by Ser214 phosphorylation might be involved in regulation of synaptic strength (Illenberger et al., 1998; Wang et al., 2003). In AD, tau redistribution from axons to somatodendrites showed mistargeted tau traffic to the correct subcellular compartments (Gotz et al., 1995).

1.3.5 Tau aggregation and degradation

Among the post-translational modifications of tau mentioned above, phosphorylation, cleavage, ubiquitination and nitration are promoting tau aggregation. Scientists debate whether tau aggregation has a toxic or a protective role inside the neuron (Cowan and Mudher, 2013). Studies show that hyperphosphorylation of tau occurs before its cleavage (Mondragon-Rodriguez et al., 2008a; Mondragon-Rodriguez et al., 2008b) and that tau cleavage takes place before NFT formation (Rohn et al., 2002). These results may suggest that abnormal phosphorylation is the key event triggering pathological aggregation of tau in AD. Both PHF isolated from AD brains and hyperphosphorylated tau have been found to block degradation and disrupt cellular homeostasis (Keck et al., 2003; Ren et al., 2007),

suggesting that PHF is an important mediator in cell toxicity and cellular regulation. Hyperphosphorylation facilitates tau's aggregation and the formation of tau aggregates may block the intracellular trafficking of functional proteins, which eventually causes deficits in axonal and dendritic transport in the neurons (Johnson and Stoothoff, 2004). Intracellular compartments that are essential for normal metabolism are interrupted by PHF-tau.

The results obtained by Morsch et al. suggest that AD patients with NFTs could live for a long time due to "protective" effect from tau aggregation (Morsch et al., 1999). However, these neurons are eventually broken up and the intracellular tau aggregates are released to the extracellular space (Bondareff et al., 1989; Cras et al., 1995). Such tau aggregates are termed, ghost tangles (Bancher et al., 1991). Broken neurons not only generate tau aggregates into the extracellular space to form extracellular NFT but also generate other cytoplasmic forms like monomeric tau that in turn can enter the surrounding neurons and promote tau aggregation (Michel et al., 2014). Interestingly, aggregated tau did not have the same toxic effect. Taken together, it has been implied that aggregation of tau or hyperphosphorylation of tau could be toxic for the neurons and lead to the formation of NFTs in some neurons. Thus, the damaged neurons with or without NFTs could die, a consequence of which is that intracellular tau becomes an extracellular species, and intracellular NFTs become extracellular ghost tangles. In summary, hyperphosphorylated tau may be a toxic agent for neurons, as intracellular tau aggregates are ineffective in their protective role. Also extracellular tau could be toxic for the surrounding neurons that are participating in spreading the tau pathology.

The ubiquitin-proteasomal system and endosome-autophagy-lysosomal system are the major proteolytic systems that clear the damaged, misfolded and aggregated proteins (Lee et al., 2013). Proteasome dysfunction might initiate the process of tau aggregation, and might be the consequence of AD, where PHF-tau is likely to be resistant to proteasomal degradation (Ren et al., 2007). The activity of the two degradation systems were found to be up-regulated in AD brains (An et al., 2003; Nixon et al., 2005). While the ubiquitin-proteasomal system is mostly responsible for the degradation of short-lived proteins, autophagy-lysosomal system is accountable for clearing many long-lived proteins and damaged organelles (Schreiber and Peter, 2014). Interestingly, recent data suggest that tau aggregation resulted due to autophagy deficiency may be a major contributor to the pathology (Chesser et al., 2013).

1.4 Mammalian target of rapamycin

1.4.1 mTor structure

The mammalian target of rapamycin (mTor) is a 289 kDa serine/threonine protein kinase that plays a critical role in the regulation of several intracellular processes, such as mRNA transcription and translation, cell growth and proliferation, autophagy, cell survival and cytoskeletal organization (Huang and Fingar, 2014; Takei and Nawa, 2014). mTor contains 2,549 amino acids and is composed of several conserved structural domains (illustrated in Figure 4), including: N-terminal, HEAT (Huntington, Elongation factor 3, a subunit of protein phosphatase 2A, and the yeast Tor) repeats, FAT domain (FKBP-associated protein (FRAP), ataxia telangiectasia (ATM), transactivation/transformation domain-associated protein (TRRAP), the FRB (FKBP12-rapamycin-binding) domain that allows the interaction for FKBP12-rapamycin binding, a PIKK (PI3-kinase-related kinase) domain, a regulatory domain (RD), and a FATC (FAT, C-terminal) domain (Baretic and Williams, 2014; Maiese et al., 2013).

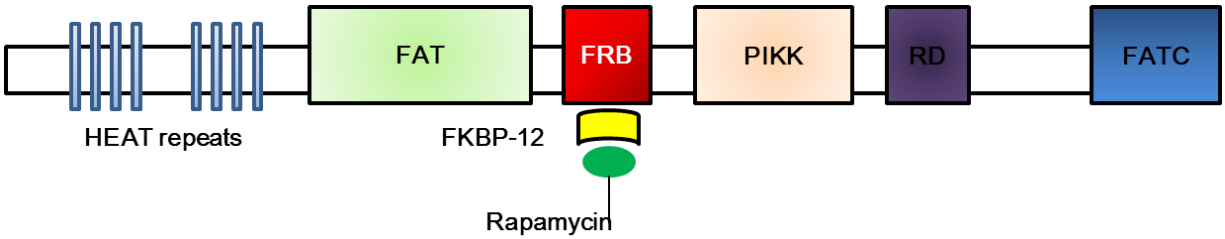

Figure 4. Schematic figure representing the structural domains of mTor kinase. mTor consists of 6 structural domains: HEAT (Huntington, Elongation factor 3, A subunit of protein phosphatase 2A and the yeast Tor) repeats, FAT domain (FKBP-associated protein (FRAP), ataxia telangiectasia (ATM), transactivation/transformation domain-associated protein (TRRAP)), the FRB (FKBP12-rapamycin-binding) domain that allows the interaction for FKBP12-rapamycin binding, a PIKK (PI3-kinase-related kinase) domain, a regulatory domain (RD) and a FATC (FAT, C-terminal) domain. Adapted from (Yang et al., 2013) .

1.4.2 mTor complex, regulation and function

In cells, mTor exists in two distinct protein complexes: mTor complex1 (mTorC1) and complex2 (mTorC2). In addition to mTor, both complexes contain mammalian lethal with SEC13 protein 8 (mLST8/also known as GβL) and DEP domain-controlling mTor interacting protein (DEPTOR). Furthermore, mTorC1 consists of regulatory-associated protein of mTor (RAPTOR) and 40 kDa Pro-rich Akt substrate (PRAS40). mTorC2 is further composed of rapamycin-insensitive companion of mTor (RICTOR), mammalian stress-activated protein

kinase interacting protein 1 (mSIN1) and protein observed with RICTOR1 (PROTOR1) (Huang and Fingar, 2014; Takei and Nawa, 2014) (Figure 5).

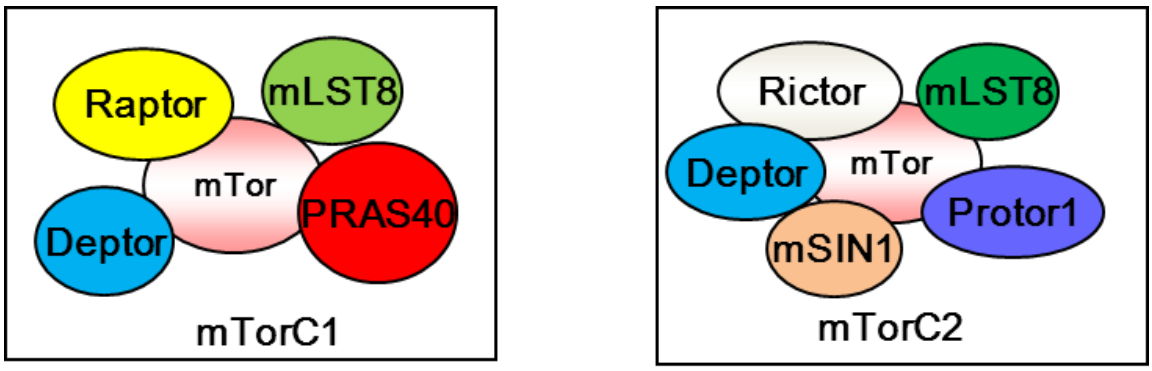

Figure 5. Schematic figure representing mTorC1 and mTorC2. mTor complex1 (mTorC1) is composed of: mTor, mammalian lethal with SEC13 protein 8 (mLST8), DEP domain-controlling mTor interacting protein (DEPTOR), regulatory-associated protein of mTor (RAPTOR) and Pro-rich Akt substrate (PRAS40). mTor complex2 (mTorC2) comprises: mTor, mLST8, DEPTOR, rapamycin-insensitive companion of mTor (RICTOR), mammalian stress-activated protein kinase interacting protein 1 (mSIN1) and protein observed with RICTOR1 (PROTOR1). Adapted from (Yang et al., 2013)

mTorC1 is activated by nutrient availability, growth factor signaling, and cellular stress levels via the phosphoinositide 3-kinase (PI3K)/v-Akt murine thymoma viral oncogene homolog-1 (Heras-Sandoval et al., 2014; Maiese et al., 2013) and the Ras/extracellular signal regulated kinase 1 and 2 (ERK1/2) pathways; while it is inhibited by deficient energy as well as nutrient deprivation via the 5-adenosine monophosphate-activated protein kinase and glycogen synthase kinase-3(GSK-3) pathway (Garelick and Kennedy, 2011). whereas, PKA can up-regulate mTorC1 via activating ERK1/2. mTorC1 exerts its functional effects by directly phosphorylating its downstream regulators of protein translation, namely the p70S6 kinase (S6K), 4E binding protein 1 (4E-BP1) and the eukaryotic elongation factor 2 (eEF2), which in turn can influence a variety of processes, including protein homeostasis, cell growth, proliferation, metabolism and autophagy .

mTorC2 is less characterized on the basis of recent data that sheds light on some of its functions that implicate mTorC2 in the regulation of cytoskeleton, as well as in the modulation of cell survival via phosphorylating its downstream substrate AKT .

1.4.3 mTor signaling in AD

Dyshomeostasis of mTor signaling is implicated in neurodegenerative diseases (Wang et al., 2013) (Figure 6). The levels of p-mTor and its downstream targets p70S6K, 4E-BP1 and eEF2 are increased in postmortem human AD brains compared to control brains. Also up-regulation of mTor and p70S6K was found to be associated with accumulation of hyperphosphorylated tau in NFTs in AD (An et al., 2003; Li et al., 2005; Li et al., 2004). A significant higher level of p-p70S6K in homogenates from the medial temporal cortex of AD patients (n=22) when compared to control brains (n=13), and an increased level of p-p70S6K in neurons known to develop NFTs at later stages were found (An et al., 2003). By sampling the same set of tissues, we have also found dramatically increased levels for p-eIF4E, p-4E-BP1 and p-mTor (S2481) in AD brains (Li et al., 2005; Li et al., 2004; Pei and Hugon, 2008). Some of these proteins are downstream targets of mTor. Others also have found that the ratio of p-mTor S2448 / total mTor increases 2.6-fold in AD when compared to control brains (Griffin et al., 2005). Moreover, inhibition of mTor with rapamycin improves learning and memory and reduced Aβ and/or tau pathology in 3xTg-AD models (Caccamo et al., 2013; Caccamo et al., 2010). Inhibition of mTor is associated with increased autophagy in 3xTg-AD models, with improvement in memory functions and decrease in Aβ levels (Caccamo et al., 2013; Caccamo et al., 2010). Loss of mTor signaling has been shown to impair long-term potentiation and synaptic plasticity in AD models (Ma et al., 2010). Taken together, these data suggests that increased mTor activity is of note in AD pathology.

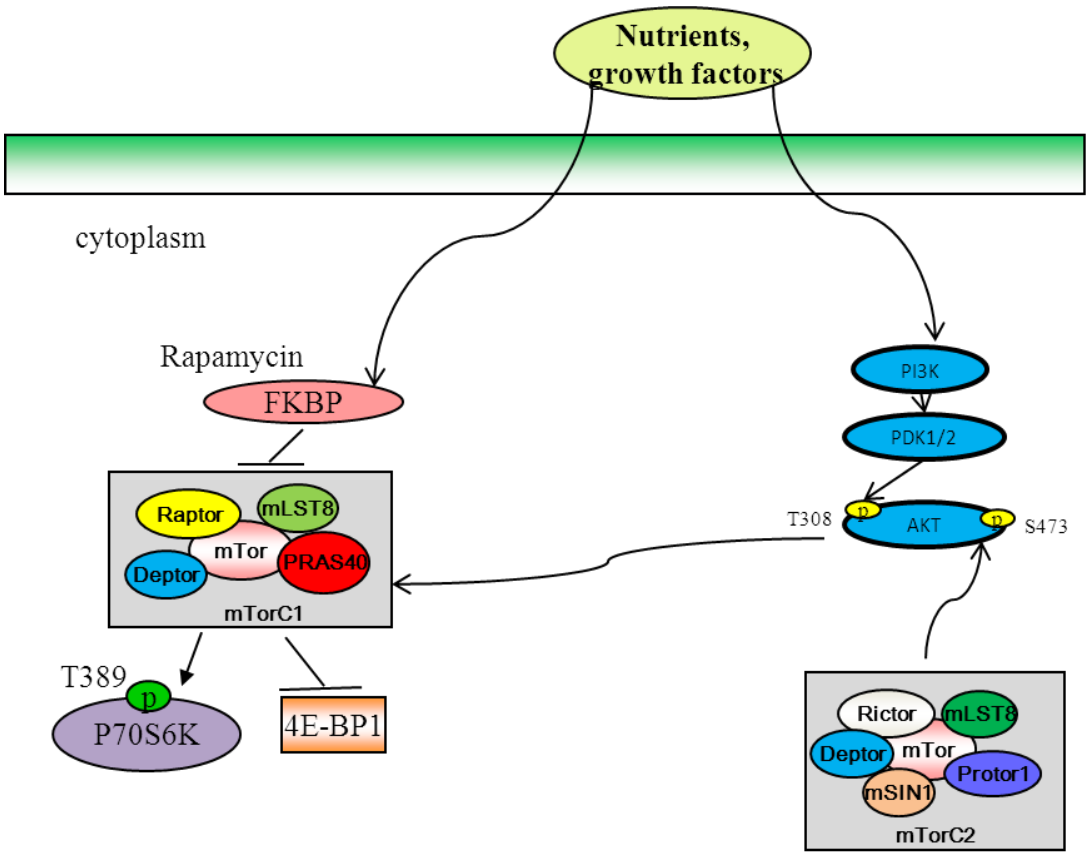

Figure 6. Regulation of the mTor signaling. mTorC1 is activated by receptor signaling nutrients, growth factor signaling through the PI3K–Akt pathway. Through its downstream effectors the 4E-BP1 and p70S6K, mTORC1 controls neuronal protein synthesis. mTorC2 regulates neuronal actin cytoskeleton dynamics via its substrate AKT. Adapted from (Huang and Fingar, 2014)

1.4.4 mTor and tau protein

Evidence from postmortem studies using human AD brains demonstrate a link between mTor signaling and the development of tau neuropathology (Pei et al., 2006). The mTor signaling is thought to be involved in the translation of tau via 5'top mRNA, suggesting that up-regulated mTor signaling mediates continuous tau synthesis in degenerating neurons through its downstream target p70S6K. It has been shown that *in vitro* p70S6K directly phosphorylated tau at S262, S214 and T212 sites (Pei et al., 2006).

Treatment of primary cultured neurons of rat cortical cortex and SH-SY5Y cells with 100 μM zinc induced an increased level of phosphorylated tau (An et al., 2005; An et al., 2003). Pretreatment with rapamycin, however, can attenuate the effects induced by zinc. Treating wild type mouse neuroblastoma cells with Aβ25-35 demonstrated a parallel increase in p70S6K activation and tau phosphorylation (Zhou et al., 2008). It was reported that inhibiting S6K and the PI3K signaling pathway activates PP2A and GSK-3β, balancing tau phosphorylation (Liu et al., 2008).

It has been shown that inhibition of mTor activity with rapamycin suppresses neurodegeneration in the brains of flies in Drosophila tauopathy models overexpressing wild type or mutant human tau (tau R406W) (Khurana et al., 2006). Thus mTor may enhance tau-induced neurodegeneration in a cell cycle-dependent manner, while pharmacologic or genetic reduction of mTor activity may rescue it.

1.4.5 mTor and autophagy

As mentioned above, mTorC1 regulates autophagy, a conserved pathway and a major process for delivering long-lived proteins and organelles to lysosomes for degradation (Diaz-Troya et al., 2008). Under stress conditions or nutrient deficiency, autophagy is considered to be essential for cellular homeostasis, development, and growth via degrading cytosolic materials, in order to either remove toxic components for cell survival or to supply alternative energy metabolism pathways. Therefore, alteration of autophagy is associated with several cellular pathologies and neurodegenerative diseases (Cuervo et al., 2005; Nixon et al., 2005). Autophagy can be generally classified into three types: 1) chaperone-mediated autophagy (Wang and Mao, 2014), which involves distinct chaperones selectively assisting in delivering cytoplasmic proteins with a KFERQ-like motif into lysosomes via the lysosomal-membrane protein type 2A (LAMP-2A); 2) microautophagy, in which cytoplasmic protein are directly engulfed into the lysosome; 3) macroautophagy (referred to as autophagy), the most characterized form of autophagy, in which portions of the cytosol and organelles (such as mitochondria) are sequestered into a double-membrane autophagic vacuole that subsequently fuses with lysosomes for protein or organelle degradation (Wang and Mao, 2014). Although the molecular mechanisms of autophagic process is still poorly understood in mammalian cells, more than 30 such genes involved in autophagy (Atg) have been discovered in mammals. Sixteen genes (Atg 1-10, 12-14, 16 and 18) are required for forming the isolation membrane and the autophagosome (Mizushima et al., 1998; Suzuki et al., 2001). Nucleation and elongation of the isolation membrane are two major steps in the process of autophagosome formation. The ULK/Atg1 kinase complex, the autophagy specific PI3-kinase complex, and phosphatidylinositol 3-phosphate (PI(3)P) complex and their related proteins are important for the nucleation, while the Atg12- and Atg8- conjugation systems play important roles in its elongation. Other proteins are also required for the autophagosome-lysosome fusion, as well as lysosomal acidification and digestion, and other regulatory signals (Diaz-Troya et al., 2008; Jung et al., 2009). Microtubule-associated protein light chain 3 (LC3) is a mammalian homolog of yeast, Atg8, localized in the autophagosome membrane. LC3 is necessary for the formation of autophagosome and participates in the

formation of autophagosomal membrane. After synthesis, LC3 is cleaved by Atg4 at C terminus to produce LC3-I, which is further converted to LC3-II by Atg7 and Atg3. The amount of LC3-II is correlated with the growing autophagosome membrane and is often used as a marker of autophagy induction (Kabeya et al., 2000; Kabeya et al., 2004).

The role of autophagy in AD is not completely understood and contradictory reports exist in literature. Nixon and his colleagues have found that macroautophagy is induced in human AD brain and PS1/APP mice, leading to the pathological accumulation of autophagic vacuoles (AVs) containing Aβ within affected neurons. Their data suggest that an increase in autophagy induction may lead to a further accumulation of Aβ (Boland et al., 2008; Lafay-Chebassier et al., 2005; Yu et al., 2005). In contrast, other reports show that autophagy protects neurons from Aβ toxicity (Caccamo et al., 2010; Hung et al., 2009; Ling et al., 2009; Spilman et al., 2010). Oddo's group has shown that increasing autophagic induction reduces soluble Aβ and tau levels in the brains of rapamycin-treated 3xTg-AD mice. This was indicated by a significant increase in LC3II and other autophagy related proteins, including Atg5, Atg7 and Atg12 (Caccamo et al., 2009; Caccamo A, 2011; Caccamo A, 2010). Supporting this view, autophagy induction correlates with the decrease in Aβ levels in another AD mouse model, hAPP J20 (Spilman et al., 2010). Results from Paul Greengard's group show that employing a small-molecule enhancer of rapamycin to induce autophagy in immortalized cell lines and primary neurons led to an 80% reduction in Aβ40 and Aβ42 levels (Tian et al., 2011).

Taking these results together, it is plausible to assume that increasing autophagy induction in the early stages of AD would facilitate autophagosomal formation, resulting in increased Aβ and tau clearance; whereas, increasing autophagy induction in late stages of AD further blocks the cells by producing excess autophagosomes that will not be cleared (Oddo, 2012).

2 AIMS OF THE THESIS

The main aim of this thesis was to examine the role of mTor in the pathogenesis of AD with a special focus on its effects on tau protein. In vitro cellular models and postmortem human brain tissues provided model systems used in our investigations.

The specific aims of the thesis were:

1. To explore the homeostatic relationship between mTor and tau.
2. To characterize the role of mTor in cell survival.
3. To investigate the involvement of mTor in tau localization and secretion.
4. To study how mTor would affect cell growth and proliferation.

3 MATERIALS AND METHODS

3.1. Materials

This section contains a general discussion of the methods and model systems used in this thesis. Detailed descriptions of each experimental procedure are provided in the respective papers.

3.1.1 Plasmids and reagents

For the descriptions of plasmids and reagents, please see the section of the materials and methods in **Papers I, II, III, IV.**

3.1.2 Antibodies

For the description of antibodies used in this thesis, please see the section of materials and methods in **Papers I, II, III, IV.**

3.2. Postmortem human brain tissue

Postmortem human brain material from both AD and non-demented control patients were used for Paper I and Paper III, where further description can be found. Tissues were obtained from the Netherlands Brain Bank. Permission to use this tissue in experimental procedures was granted by the Ethics Committee Review Board at Karolinska Institutet (157/02).

3.3. Cell culture

The stable human SH-SY5Y neuroblastoma cells that carry various transgenes of mTor or S6K were established in **Paper I**, and used in **Papers II-IV** as well. For the majority of the experiments, the stable transfected cells were grown to 70–80% confluence in 100 mm culture dishes, employing Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1: 1) supplemented with 10% fetal bovine serum (FBS). The cells were then cultured in 1% FBS media for 24h, and experiments were performed in serum deprived conditions from 30 min to 8h before cells were harvested. As cited in **Paper I**, the physiological (100 μM) and pathophysiological (300 μM) doses of zinc were described in our previous article (An et al., 2005). In **Paper IV**, stable transfected SH-SY5Y cells were differentiated with 10 μM retinoic acid for 5 days and 0.5-5ng / ml brain-derived neurotrophic factor (BDNF).

3.4. Immunostaining

3.4.1 Immunohistochemistry & immunofluorescence for human brain tissues

For the description of postmortem human brain materials, and the methods of immunohistochemistry and immunofluorescence, please see the section of the materials and methods in **Paper I or Paper III**.

3.4.2 Immunofluorescence for cells

For the preparation of cells and immunofluorescence method, please see the section of the Materials and Methods in **Paper III**.

3.4.3 Immunoelectron microscopy (EM)

For the preparation of cells and immunoelectron microscopy, please see the section of the Materials and Methods in **Paper III**.

3.5. Sample preparations

Cell lysates were sonicated on ice and centrifuged at 1,000 –12,000 × g at 4 ℃ for 10–20 min to collect supernatants free of nuclei and large cell debris. Soluble fraction of tau, free of microtubules and insoluble fraction of tau containing microtubules, were prepared as described in **Paper I**. Cytosolic fraction, membrane fraction, endoplasmic reticulum, mitochondria, Golgi membrane and exosome fractions were prepared as described in **Paper III**. Purified proteins from culture condition media were isolated as described in **Paper III**.

3.6. Protein measurement, Western blotting and Dot blotting

Protein concentration of samples in this thesis was prepared from stable cell lines and was determined by bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) (**Papers I- IV**). Western blotting were used in **Papers I- IV**, and dot blotting in **Paper III**.

3.7. Other methods

Using mass spectrometry to identify *in vitro* tau phosphorylation sites by mTor, is described in detail in **Paper I**. Mitochondrial succinate dehydrogenase activity was measured by MTT reduction assay (**Paper II**), the level of externalized phosphatidylserine was analyzed by Flow cytometry (**Paper II**) and specific protein expression changes was identified by mass spectrometry (**Paper II)**. The rate of cell proliferation was analyzed by WST-1 method (**Paper IV**) and gene profiles analyzed by microarray are further described in **Paper IV**.

3.8. Statistical analysis

For data from MTT reduction assay, flow cytometry, western and dot blotting, statistical comparisons between different experimental groups were performed by one-way ANOVA followed by Bonferroni post-hoc test analyses (**Papers I, II, III**). For the enrichment analysis, the differences between groups was evaluated by the paired student t-test in Excel (**Paper II**). For data from WST-1 and microarray analyses, the differences between experimental groups were evaluated by paired student t-test (**Paper IV**). A value of $p \leq 0.05$ was considered as significant.

4 RESULTS AND DISCUSSION

This section summarizes the main findings of the thesis based on **Papers I-IV**. Figures and more details concerning the results are found within the respective papers.

4.1 Up-regulated mTor in AD brain mediates tau protein synthesis and phosphorylation

Tau is a substrate for several kinases studied *in vitro* and *in vivo*. The active forms of some kinases, such as GSK-3β, MAPK, p70S6K and PKB, have been shown to have increased immunoaggregates in tangle-bearing neurons in AD compared with control neurons (An et al., 2003; Chin et al., 2000; Pei et al., 1999; Pei et al., 2002; Pei et al., 2003b). Our previous article showed that p70S6 kinase, the well characterized immediate downstream target of mTor, regulated the synthesis and phosphorylation of tau (Pei et al., 2006). We previously had found a ~3-fold increase of p-mTor (S2481) but no changes for p-mTor (S2448) in the homogenates of AD brains using dot blots (Li et al., 2005). To further investigate the status of p-mTor (S2448) in AD brains, we have employed double immunostaining of tau and mTor in AD brains, and have found increased aggregation of p-mTor (S2448) in pyramidal neurons that accumulate hyper-p tau S422 and PHF-1, respectively (**Paper I**). Our data indicates that up-regulated mTor is significantly linked to PHF-tau and AD pathology.

Knowing that tau is mostly phosphorylated at serine/threonine (S/T) residues, and that mTor is an S/T kinase, we hypothesized that mTor would also be directly involved in phosphorylating tau. Exploring this, we combined *in vitro* phosphorylation assay with mass spectrometry. We have shown for the first time that mTor could directly phosphorylate tau at S214, T231, and S356 (**Paper I**). This is a group of epitopes shared by S6K and Akt signals (Ksiezak-Reding et al., 2003; Pei et al., 2006; Virdee et al., 2007), which regulate tau phosphorylation sites at flanking regions and microtubule repeat regions, resulting in disruption of microtubule binding (Goode and Feinstein, 1994; Gustke et al., 1992). In gene modified SH-SY5Y cells，we have found that overexpressed mTor increased total tau, de-p-tau and p-tau, while silenced mTor, or the inactive form of mTor and S6K, decreased total tau, de-p-tau and p-tau. These results support the possibility suggested by our group that increased tau might result from the up-regulated translation of tau mRNA, which has the 5' TOP structure playing a regulator role in translation control (Pei et al., 2006). In human neuroblastoma cells carrying overexpressed (m-WT) or silenced (m-SR1 and m-SR2) mTor mutation we found p-tau S214 or S356 in 1% Triton-X-100 insoluble fraction, the two epitopes of tau having been phosphorylated by mTor *in vitr*o, suggesting that mTor might mediate the conversion process of tau protein from soluble hyperphosphorylated form into

insoluble aggregate form. Results presented in **Paper I** show that up-regulated mTor increases the phosphorylated levels of Akt and GSK-3β, the levels of both total and phosphorylated levels of PP2A, and the protein levels of both PKA α and PKA β; but the level of the PI3K-mTorC1 downstream target cdk5 is decreased. Changes in mTor activity by genetic modification in SH-SY5Y cells cause fluctuation of these enzymes, suggesting that the endpoint of the phosphorylation status of tau in neurons in AD brains is itself synergistically mediated by numerous upstream or downstream mTor signals. Taking together, these data suggest that mTor modulates the balance of tau synthesis and phosphorylation required for neurons to maintain physiological function.

4.2 Role of mTor in cell survival

mTor is involved in the regulation of both cell survival and protein synthesis (Huang and Fingar, 2014; Takei and Nawa, 2014). In **Paper I**, we found that the catalytic subunit of mTor phosphorylates tau *in vitro*. Also mTor in human neuroblastoma cells, when genetically modified, can induce essential biochemical changes, such as translation, phosphorylation, and aggregation of tau. In **Paper II**, we focused on molecular events involved in pro-survival mechanisms mediated by mTorC2. In this study, we employed the same genetically modified mTor cell models as in **Paper I**. After 4.5h of serum deprivation of SH-SY5Y cells, we have observed that approximately 60% of the cells were in various states of apoptosis in its different stages. Employing flow cytometry (FACSCalibur™), we observed that up-regulated mTor increased the percentage of viable cells but decreased that of apoptotic cells; while down-regulated mTor dramatically reduced viable cells and increased the number of apoptotic cells. Interestingly, S6K knockdown did not cause a significant change in cell viability in either early or late apoptosis. mTorC2 was recently identified as PDK2 kinase (Cota, 2014). Taken together, this might suggest a direct regulatory role of mTorC2 in cell survival via phosphorylation and activation of Akt (Zoncu et al., 2011), whilst mTorC1 seems not to be involved in pro-survival signals.

Using mass spectrometry, we have showed that genetic modification of mTor leads to numerous changes of protein expression. Both proteomic analysis and western blotting show that up-regulated mTor increases levels of Annexin A5, 14-3-3 protein zeta/delta, cofilin 1, and mortalin; while down-regulated mTor results in correspondingly decreased levels of anti-apoptosis related proteins. Levels of two caspase inhibitors, thioredoxin-dependent peroxide reductase and peroxiredoxin-5, were increased in cells expressing up-regulated mTor, and decreased in cells where mTor was down-regulated. Both thioredoxin-dependent peroxide reductase and peroxiredoxin-5 have anti-oxidant functions, and are located in mitochondria.

These results locate the direct involvement of mTorC2 in cell survival by these up-regulating proteins in the caspase inhibitory pathway and anti-apoptosis functional pathways. This also suggests that up-regulated mTorC2 may have an important role in promoting cell survival by protecting cells from immediate apoptosis that otherwise might follow after the aggregation of toxic phospho-tau (promoted by mTorC1).

4.3 Role of mTor in tau distribution and trafficking

In AD, the localization of tau, particularly the redistribution of hyperphosphorylated tau, is altered, suggesting that defective tau trafficking to the various subcellular compartments might be a pathological hallmark during early tauopathic development (Mandelkow and Mandelkow, 2012). In **Paper I**, we have found that mTor mediates the biochemical processing (translation, phosphorylation, and aggregation) of tau in SH-SY5Y cells with different genetic modifications of mTor activity (**Paper I**). In both **Paper I and Paper III**, we have found that the increased level of tau is driven by up-regulating mTor. Furthermore, we have found that up-regulated mTor increased non-phosphorylated tau (Tau-1) bound to membrane and phosphorylated tau (PHF-1 and TG3) in cytosol (**Paper III**). Contrarily, down-regulated mTor decreased non-phosphorylated tau (Tau-1) in membrane fractions and phosphorylated tau (PHF-1 and TG3) in cytosol, suggesting that mTor regulates tau synthesis and phosphorylation, and that it could be involved in the regulation of tau localization. To investigate if macroautophagy is involved in intracellular tau deposition, double immunostaining was used, which showed that the co-localization of Np-tau and p-tau with LC3 positive vacuoles is significantly higher in overexpressed mTor SH-SY5Y cells than control cells. These results suggest that high levels of mTor signaling (hence low levels of autophagy) raise mTor gain-of-function that occurs during aging, a process that may facilitate the development of tau pathology (Oddo, 2012). Moreover, we found that increased levels of tau species are found in purified fractions derived from conditioned culture media of overexpressed mTor SH-SY5Y cells, while no significant change were detected in exosomal fractions. We have demonstrated that intracellular tau is partially localized to different cellular organelles, including autophagic vacuoles, endoplasmic reticulum, mitochondria and Golgi apparatus in AD brains. Moreover, we found increased aggregation of tau partially localized with subcellular compartments in overexpressed mTor SH-SY5Y cells (**Paper III**). On the basis of these data, we suggest that mTor may mediate the secretion of tau into extracellular space using an alternative exosome independent pathway in SH-SY5Y cells. Overall, our data suggests that mTor is involved in autophagic processing and tau traffic, an improvement in understanding the role of mTor.

4.4 Effect of silenced mTor in cell growth and proliferation

mTor is a downstream effector of Akt that regulates cell growth and proliferation (Lawlor and Alessi, 2001; Song et al., 2005), highlighting the importance of mTor can be a clinically potential drug target in regulating cellular growth and proliferation in various cancers and other diseases (Laplante and Sabatini, 2012; Lewis et al., 2000; Rayess et al., 2012; Wang et al., 2013). Rapamycin, an mTor inhibitor, has been found to lower cell growth and proliferation, and prolong lifespan (Laplante and Sabatini, 2012; Maillet et al., 2013). mTor also acts as a key regulator of nutrient-dependent pathways that coordinate mRNA translation.(Fang et al., 2001). However, it is unclear how mTor exerts its effects and what gene detailed mechanism(s) are utilized in cell growth and proliferation of differentiated SH-SY5Y cells. In **Paper IV**, we explored WST-1 assay and found that the suppression of mTor significantly decreases cell growth and proliferation in differentiated SH-SY5Y cells at different time points. Western blotting demonstrated that silenced mTor reduced levels of total mTor and p-mTor S2448 while it also suppressed the expression of p-AKT S473 and p-S6K T389. Microarray was employed to specifically target mTor in cells and resulted in 716 differentially expressed genes being found, among which 27 were up-regulated genes and 49 down-regulated genes that link to cell growth and proliferation. Our newly detailed evidence demonstrates that mTor enhances differentiated SH-SY5Y cell growth and proliferation by not only activating AKT-mTor-S6K signaling pathway, but also directly or indirectly by affecting key proteins, such as Bcl2, CDK4 inhibitor, various interleukins, clusterin, 24 dehydrocholesterol reductase or the TGF beta superfamily (Harold et al., 2009; Hausmann et al., 2011; Lambert et al., 2009; Vela et al., 2002). Taken together, the data provide novel genomic evidence that mTor promotes cell growth and proliferation via the AKT-mTor-S6k pathway.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

During the last decades, a major effort has been devoted to understand the biological mechanisms behind AD, as well as the role of tau phosphorylation in order to find a cure for the disease.

Previously, our group has extensively studied the interrelations between mTor signalling and tau hyperphosphorylation in human and murine neuroblastoma cells, rat primary neurons, and metabolically active rat brain slices that were treated with a physiological or pathological dosage of zinc (An et al., 2005; An et al., 2003; Liu et al., 2008; Pei et al., 2003a). In the present thesis, we have focused on studying in detail the role of mTorC1 and mTorC2 in causing biochemical changes, such as: translation, phosphorylation and aggregation of tau; the molecular events behind pro-survival mechanisms; tau trafficking; as well as modulations occurring in cell growth and proliferation. We have chosen post-mortem human AD and control non-demented brains, as well as human neuroblastoma cells, employing various genetic modifications of mTor activity as our model systems.

We have found that p-mTor S2448 accumulates in tangle-bearing neurons and that it mediates tau phosphorylation at T231, S214 and S356 *in vitro*. We have also shown that mTor mediates tau synthesis and its deposition, resulting in compromised microtubule stability. Changes in mTor activity have been shown to cause fluctuation in the levels among a battery of tau kinases, such as PKA, AKT, GSK-3β, Cdk5 and tau protein phosphatase PP2A. These data imply that up-regulated mTor promotes tau dyshomeostasis by mediating the synthesis, phosphorylation, and deposition of tau protein.

Up-regulated proteins in the caspase inhibitory pathway and in the anti-apoptosis functional pathway provide direct evidence that mTorC2 mediates cell survival. This suggests that up-regulated mTorC2 have a beneficial role in promoting cell survival, protecting cells from the immediate apoptotic death that might result from the accumulation of toxic phospho-tau (promoted by mTorC1). This pro-survival mechanism suggests that up-regulated mTorC2 might play an important part in promoting cell survival by suppressing the mitochondria-caspase-apoptotic pathway *in vitro*.

Both in AD and in cellular models, we have shown, in agreement with previous findings, that tau was localized within different organelles (autophagic vacuoles, endoplasmic reticulum, Golgi complexes, and mitochondria). We have found that mTor is directly or indirectly linked to the synthesis and distribution of intracellular tau. Genetic variance of mTor (overexpression or lacking of its expression) was responsible for the altered balance

of phosphorylated/ non phosphorylated tau in the cytoplasm and different cellular compartments, thus, facilitating tau deposition. Up-regulated mTor activity resulted in significant increase in the amount of cytosolic tau, as well as correlating with its localization in exocytotic vesicles in exosomes independent pathway. Our data suggests that mTor is involved in regulating tau distribution in various subcellular organelles and in the initiation of tau secretion to extracellular space, which provides better understanding of the role of mTor in tauopathies.

Studies employing microarray analyses revealed that various genes involved cell growth and proliferation were differentially expressed in differentiated SH-SY5Y cells. The genomic evidence seems to prove that silenced mTor inhibits differentiated SH-SY5Y cell growth and proliferation not only by activating the AKT-mTor-S6K signaling pathway, but also by directly or indirectly affecting key proteins, such as Bcl2, CDK4 inhibitor, various interleukins, clusterin, 24 dehydrocholesterol reductase or the TGF beta superfamily.

As discussed above, a large number of factors and their interplay are important in the development of AD. This thesis sheds light on the role of mTor in various biochemical and molecular modifications that affect tau, as well a role in the entangled pathogenesis of AD. Our hypotheses based on the results from **Papers I-IV** are summarized in Figure 7. Further studies are needed to fully understand the molecular mechanisms linking mTor to tau *in vitro* and *in vivo*, such as how mTor interacts with different tau molecules, or how mTor mediates the formation of tau isoforms in the present cell models; and how mTor mediates tau patholgy in our mTor transgene mice. Furthermore, an open question is whether mTor is involved in the synthesis, deposition and degradation of the other key hallmark of AD - the β-amyloid.

Researchers all over the world are trying to find new treatment strategies for AD. mTor modulators have much potential, however much more investigation is needed before mTor-based therapies would represent a significant drug target for AD.

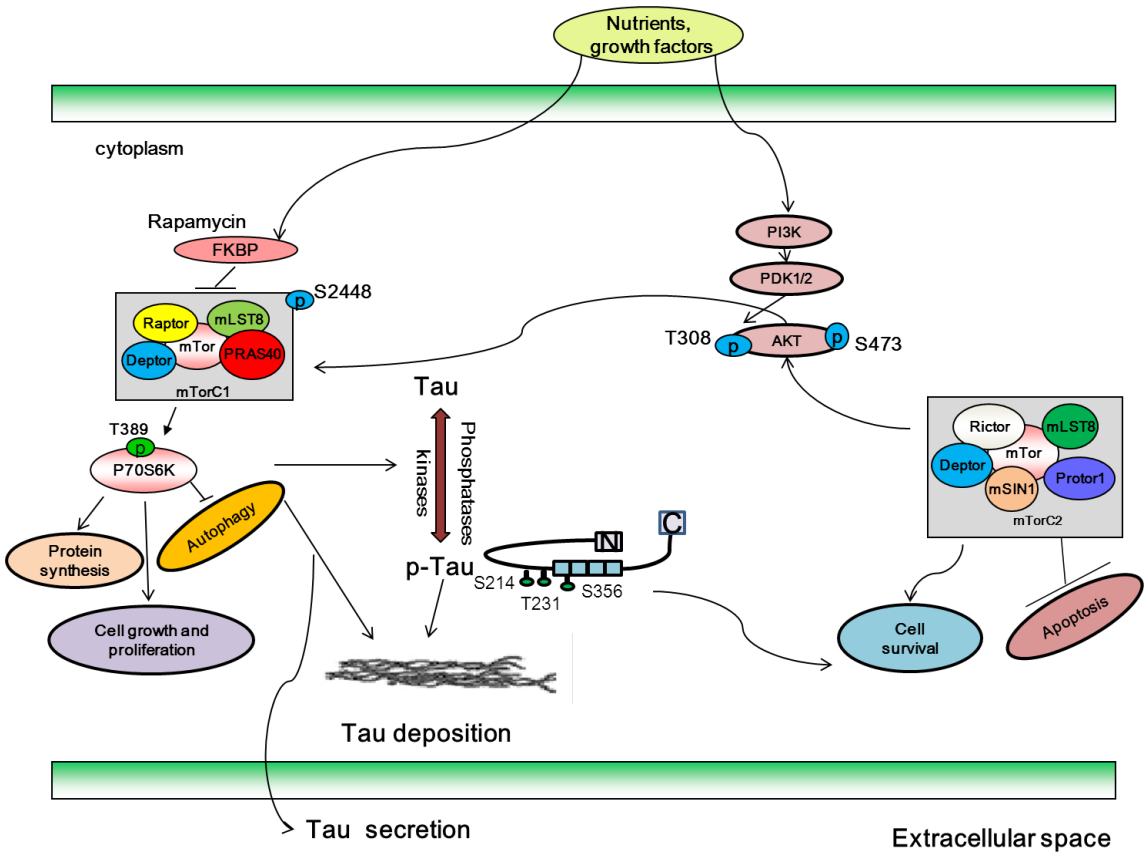

Figure 7. Summary of the results presented in this thesis. mTorC1 regulates tau protein changes (translation, synthesis, phosphorylation, and deposition) and secretion. mTorC1 inhibits autophagy by activating p70S6 kinase, and mTorC2 is a core component of the PI3K-AKT pathways that stimulates cell survival and inhibits apoptosis. Both mTorC1 and mTorC2 regulate cell growth and proliferation via the Akt-mTor-S6k pathway. Modified from (Tang et al., 2014).

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